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Endothelin-converting enzymes degrade α-synuclein and are reduced in dementia with Lewy bodies

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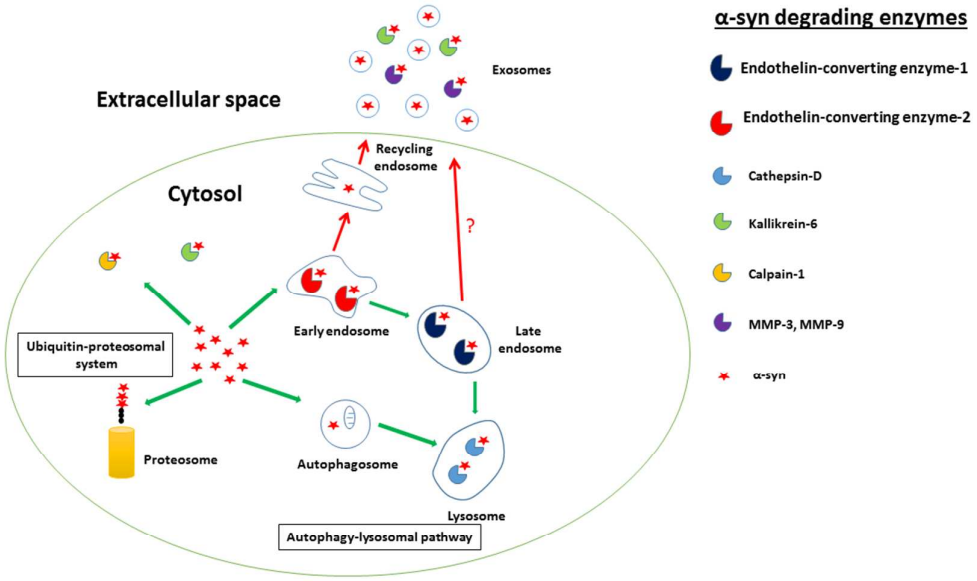
List of abbreviations

ECE-1	endothelin-converting enzyme 1
ECE-2	endothelin-converting enzyme 2
EDN1	endothelin-1
α-syn	alpha-synuclein
α-syn-P129	alpha-synuclein phosphorylated at serine-129
Aβ	beta-amyloid
DLB	dementia with Lewy bodies
AD	Alzheimer's disease
KLK6	kallikrein-6
CAPN1	calpain-1
CTSD	cathepsin-D
M6PR	mannose-6-phosphate receptor
Rab5	small Rab GTP(guanosine triphosphate)ase-5
Rab7	small Rab GTP(guanosine triphosphate)ase-7
EEA1	early endosome autoantigen-1
NAC	non-amyloid beta component



In this issue. Defective clearance, rather than increased production, most likely contributes to intracellular accumulation of α-synuclein in Lewy body diseases, including dementia with Lewy bodies (DLB) and Parkinson's disease (PD). Several enzymes are capable of degrading α-synuclein (as shown) and are often dysregulated in DLB and PD. Here we show for the first time that recombinant human α-synuclein is cleaved by endothelin-converting enzyme-1 and -2 (ECE-1/-2) and siRNA-mediated knockdown of ECE-1 or -2 increased intracellular and extracellular α-syn within SH-SY5Y cells. ECE-1 and -2 co-localise with discrete pools of intracellular α-synuclein that are implicated in the spread and propagation of Lewy body pathology. Finally, we show that ECE-1 and -2 levels are significantly reduced in post-mortem brain tissue in the cingulate cortex in DLB and correlate inversely with Lewy body pathology.

Abstract We have examined the roles of the endothelin-converting enzyme-1 and -2 (ECE-1 and ECE-2) in the homeostasis of α-synuclein (α-syn) and pathogenesis of Lewy body disease. The ECEs are named for their ability to convert inactive big endothelin to the vasoactive peptide endothelin-1 (EDN1). We have found that ECE-1 and ECE-2 cleave and degrade α-syn *in vitro* and siRNA-mediated knockdown of ECE-1 and ECE-2 in SH-SY5Y neuroblastoma cells significantly increased α-syn both intracellularly (within the cell lysate) ($P < 0.05$ for both ECE-1 and -2) and extracellularly (in the surrounding medium) ($P < 0.05$ for ECE-1 and $P = 0.07$ for ECE-2). Double immunofluorescent labelling showed co-localisation of ECE-1 and ECE-2 with α-syn within the endolysosomal system (confirmed by a proximity ligation assay). To assess the possible relevance of these findings to human Lewy body disease, we measured ECE-1 and ECE-2 levels by sandwich ELISA in post-mortem samples of cingulate cortex (a region with a predilection for Lewy body pathology) in dementia with Lewy bodies (DLB) and age-matched controls. ECE-1 ($P < 0.001$) and ECE-2 ($P < 0.01$) levels were significantly reduced in DLB and both enzymes correlated inversely with the severity of Lewy body pathology as indicated by the level of α-syn phosphorylated at Ser129 ($r = -0.54$, $P < 0.01$ for ECE-1 and $r = -0.49$, $P < 0.05$ for ECE-2). Our novel findings suggest a role for ECEs in the metabolism of α-syn that could contribute to the development and progression of DLB.

Introduction

Abnormal aggregates of α-synuclein (α-syn) within neuronal perikarya (as Lewy bodies) and neurites (as Lewy neurites) are the defining neuropathological hallmarks of Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Accumulation of insoluble (Campbell *et al.* 2000) and soluble oligomeric α-syn (Paleologou *et al.* 2009) in sporadic PD and DLB, without an increase in α-syn mRNA (Kingsbury *et al.* 2004, Wirdefeldt *et al.* 2001), suggest a major role for impaired clearance in the pathogenesis of these diseases. Dysregulation of the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP) probably contributes to the accumulation of α-syn, particularly in familial Lewy body disease (reviewed in (Deleidi & Maetzler 2012, Ebrahimi-Fakhari *et al.* 2012, Webb *et al.* 2003, Xilouri *et al.* 2013)). Cathepsin D (CTSD), the major lysosomal protease responsible for α-syn cleavage (Cullen *et al.* 2009, Qiao *et al.* 2008, Sevlever *et al.* 2008), is upregulated in PD and DLB (Yelamanchili *et al.* 2011). A small number of non-lysosomal proteolytic enzymes have been identified that cleave α-syn *in vitro*, including MMP-3, −9 (Sung *et al.* 2005), calpain-1 (CAPN1) (Mishizen-Eberz *et al.* 2003, Mishizen-Eberz *et al.* 2005) and kallikrein-6 (KLK6) (Iwata *et al.* 2003, Kasai *et*

al. 2008). These are expressed within the cytosol, or are released extracellularly, whereupon they are activated and are likely to interact with and degrade distinct pools of α-syn that are important in the accumulation and transneuronal spread of pathogenic α-syn (Luk *et al.* 2012, Mougenot *et al.* 2011, Mougenot *et al.* 2012, Masuda-Suzukake *et al.* 2013). We reported previously that the levels of CAPN-1 and KLK6 are reduced in the cingulate cortex in DLB (Miners *et al.* 2014a).

Endothelin-converting enzymes (ECE; EC number 3.4.24.71) are type II membrane metallopeptidases named for their ability to convert the inactive precursor big endothelin to endothelin-1, a potent vasoactive peptide. ECE-1 and ECE-2 share 59% sequence homology and cleave a similar range of substrates although ECE-1 is most active at a neutral pH whereas ECE-2 is most active at a mildly acidic pH (Emoto & Yanagisawa 1995). ECE-1 is expressed abundantly in endothelial cells in all organs but has also been identified within non-vascular cells within the CNS such as pyramidal neurons, cerebellar Purkinje cells and astrocytes (Naidoo *et al.* 2004, Palmer *et al.* 2010). Four isoforms of ECE-1 have been identified (ECE-1a, b, c, d): ECE-1a and ECE-1c reside within the plasma membrane (Muller *et al.* 2003, Schweizer *et al.* 1997) whereas ECE-1b and d are located within intracellular compartments including the endolysosomal system (Muller et al. 2003, Azarani *et al.* 1998, Schweizer et al. 1997). ECE-1 degrades a variety of neuropeptides (substance P, bradykinin, neurotensin, somatostatin etc) intracellularly within endosomes at a mildly acidic pH (Fahnoe *et al.* 2000, Johnson *et al.* 1999, Roosterman *et al.* 2008, Cottrell *et al.* 2009, Cattaruzza *et al.* 2009, Hasdemir *et al.* 2012). ECE-2 is mostly expressed in neural tissue and has been predominantly detected in pyramidal neurons but is also expressed in astrocytes and microglia (Palmer et al. 2010). All four ECE-2 variants are located within intracellular secretory vesicles and they too are capable of intracellular neuropeptide degradation in mildly acidic conditions (Gupta *et al.* 2015, Pacheco-Quinto & Eckman 2013). The expression and function of ECE-2 is not yet fully characterised.

ECEs hydrolyse amyloid-β (Aβ) peptide *in vitro* (Eckman *et al.* 2001) and have been implicated in the pathogenesis of Alzheimer's disease (AD). Endogenous Aβ levels were elevated in ECE knockout mice (Eckman *et al.* 2003). A recent study reported that ECEs degrade two distinct pools of intracellular Aβ: ECE-2 cleaved an intracellular pool within the autophagic and endolysosomal pathway whereas ECE-1 predominantly degraded a pool of Aβ destined for secretion (Pacheco-Quinto & Eckman 2013). In the present study, we have shown that ECE-1 and ECE-2 degrade α-syn *in vitro* and have investigated their contributions to the pathogenesis of dementia with Lewy bodies (DLB).

Materials and Methods

Study cohort

Brain tissue was obtained from the South West Dementia Brain Bank, University of Bristol, with local Research Ethics Committee approval (08/H0106/28+5). The brains had been separated midsagittally - the left half sliced and frozen at −80°C and the right half fixed in formalin for paraffin histology and detailed neuropathological examination. We examined 11 DLB and 15 control brains matched for age, gender and post-mortem (PM) delay (Table 1). The DLB group had an average age-at-death of 77.7 ± 8.9 years and a post-mortem delay of 28.2 ± 10.3 hours and consisted of 5 females and 6 males,. The controls had an average age-at-death of 80.4 ± 10.0 years and a post-mortem delay of 30.0 ± 16.8 hours and consisted of 3 females and 12 males. The MRC identifier numbers for each case are supplied in Supplementary Table 1.

Pure DLB cases were diagnosed according to consensus neuropathological criteria (McKeith *et al.* 2005). All DLB cases had Braak stage 5 or 6 Lewy body pathology (Braak *et al.* 2003). Cases were excluded on the basis of a neuropathological diagnosis of AD based on the National Institute on Aging-Alzheimer's Association guidelines for the neuropathological assessment of AD (Montine *et al.* 2012)) or the presence of a neurodegenerative disease apart from DLB. They were also excluded if there was evidence of severe cerebral amyloid angiopathy or any other significant cerebrovascular disease. Controls had no history of dementia and no neuropathological abnormalities apart from mild neurofibrillary tangle pathology (Braak tangle stage III or less) and scattered diffuse Aβ plaques in some cases.

Brain tissue

Cortical brain tissue (~200 mg), dissected from the cingulate cortex (Brodmann area 24), was homogenized in 1% NP-40 buffer (140 mM NaCl, 3 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 2 mM 1,10-phenanthroline, 0.1 M PMSF, 1.7 mg/ml aprotinin, NP-40 detergent) in a Precellys 24 homogenizer (Stretton Scientific, Derbyshire, UK) with 2.3 mm silica beads (Biospec, Glasgow, UK). The homogenates were centrifuged at 13000 rpm for 15 min at 4°C and the supernatant (soluble extract) removed, aliquoted and stored at −80°C. Insoluble pelleted material was solubilized by vigorous agitation in 6 M GuHCl (insoluble extract) and left for 4 h at room temperature (RT) before storage at −80°C.Total protein concentrations were determined using the Total Protein Kit (Sigma Aldrich, Dorset, UK) following the manufacturer's guidelines.

Assessment of cleavage of α-syn in vitro

Recombinant human full-length wild-type α-syn (5 ng) (rPeptide, Stratech, Suffolk, UK) was incubated with recombinant human ECE-1 or ECE-2 (50 – 1.56 ng) (R&D systems, Oxford, UK) at 37°C with shaking (300rpm) in a non-binding black 96-well plate. The reaction was performed either at a neutral pH (PBS or water) or in a mildly acidic assay buffer for ECE-1 (0.1 M MES, 0.1 M NaCl, pH 6.0) and ECE-2 (0.1 M MES, 0.25 M NaCl, pH 5.5). The reaction was allowed to proceed for 24 hours and samples from the reaction mix were removed at various time points (0, 5, 10, 30, 60 minutes and 2, 4, 6 and 24 hours). Reaction samples were diluted in sample buffer (0.5 M Tris–HCl, pH 6.8, 0.25% glycerol, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue) containing 5% β-mercaptoethanol and denatured by heating at 95°C for 5 min. They were electrophoresed on a 4–20% gradient pre-cast Tris–HCl gel (BioRad, Hemel Hempstead, UK) for 1 h at 150 mV alongside the Precision Plus protein Western C standards (BioRad). Proteins were subsequently transferred onto nitrocellulose membrane (GE Healthcare, UK, Limited) in a Mini Trans-Blot Cell (BioRad) at 90 mV for 1.5 hours on ice. Membranes were immersed for 1 h in 10% non-fat dried milk powder in Tris-buffered saline-Tween 20 (0.05%), washed briefly in TBS-T (15 min, room temperature, with agitation x3) and probed with mouse anti-human α-syn (syn-1) (1 in 1000) (BD Biosciences, Oxford, UK) diluted in 5% non-fat dried milk/TBS-T (1:1000, at 4°C overnight with agitation). The membrane was washed in TBS-T (3 × 15 min, with agitation) and incubated with anti-mouse peroxidase-conjugated secondary antibody (Vector labs, Burlingame, CA, U.S.A) diluted in 5% non-fat dried milk/TBS-T (1:4000, 2 h, room temperature, with agitation). Membranes were washed in TBS-T (3 × 15 min, with agitation) and immune-labelled proteins visualised using the Enhanced Chemiluminescence (ECL) Detection System (Amersham Biosciences, Buckinghamshire, UK). ECL detection reagents were applied (5 min) and membranes were visualised using the ChemiDoc XRS+ system (Biorad). Volumetric analysis of the relative band density was performed using Image Lab 5.0 software.

ECE-1 and ECE-2 sandwich ELISAs

ECE-1 protein level was measured in NP-40-soluble brain tissue extracts (total protein 10 µg) and SH-SY5Y cell lysates (total protein 30 µg) using a commercially available sandwich ELISA kit (catalogue number SEA483Hu) (USCN, Wuhan, Hubei Province 430206, P.R. China) and was interpolated from a standard curve generated from a serial dilution of recombinant human ECE-1 (2000−31.25 pg/ml). Measurements for each sample were made in duplicate. ECE-2 protein level was also measured by a commercially available sandwich

ELISA kit (catalogue number CSB-EL007372HU) (Cusabio, Wuhan, Hubei Province 430206, P.R. China). ECE-2 concentration in brain tissue extracts (total protein 25µg) and SH-SY5Y cell lysates (total protein 25µg) was interpolated from a standard curve produced by serial dilution of recombinant human ECE-2 (3000−46.8 pg/ml) and measured in duplicate.

α-syn and α-syn-P129 sandwich ELISAs

α-syn and α-syn-P129 levels were measured by sandwich ELISA in GuHCl-solubilised brain tissue extracts and SH-SY5Y cell lysates, as previously described (Miners et al. 2014a, Miners *et al.* 2014b, Swirski *et al.* 2014) For the α-syn ELISA, a mouse monoclonal anti-α-syn antibody (Syn-1, raised against epitope corresponding to aa 91–99 (Perrin *et al.* 2003) (0.5 µg/ml) (BD Biosciences, Oxford, UK) was used as the capture antibody and a biotinylated goat polyclonal anti-α-syn (raised against full-length recombinant human α-syn) (1 µg/ml) (R&D systems, Oxford, UK) was used as the detection antibody. Total α-syn concentration in brain tissue extracts (diluted 1 in 99 in PBS) and SH-SY5Y cell lysates (total protein 8 µg) was determined following interpolation from a standard curve produced from a serial dilution of recombinant human α-syn (62.5−0.97 ng/ml) (rPeptide, Stratech, Suffolk, UK).

The α-syn-P129 ELISA was performed as previously described (Miners et al. 2014a, Miners et al. 2014b). Briefly, a mouse monoclonal anti-α-syn antibody (syn-1) (0.5 µg/ml) (BD Biosciences, Oxford, UK) was used as the capture antibody and biotinylated anti-α-syn-129 (0.8 µg/ml, Abcam, Cambridge, UK) as the detection antibody. α-syn-P129 concentration was determined in brain tissue extracts (diluted 1:99 in PBS) and SH-SY5Y cell lysates (total protein 8 µg) following interpolation against a standard curve produced from a serial dilution of recombinant α-syn (200–3.25 ng/ml) (rPeptide, Stratech, UK) that had been phosphorylated at serine 129 by incubation with casein kinase II (New England Biolabs, Hitchin, UK) for 1 h at 30°C in the presence of ATP (New England Biolabs, Hitchin, UK) as previously described (Miners et al. 2014a, Swirski et al. 2014). The specificity of the ELISA for α-syn-P129 was previously demonstrated (Miners et al. 2014a, Swirski et al. 2014).

SH-SY5Y cell culture and transient siRNA-mediated knockdown of ECE-1 and ECE-2

SH-SY5Y cells (American Type Culture Collection (ATCC, CRL-2266)) and SH-SY5Y cells transfected with full-length human wild-type α-synuclein (SH-SY5Y-α-syn) were maintained in Ham's F12 nutrient mixture (F12) (42% vol/vol), Eagle's minimum essential medium (MEM) (42% vol/vol) supplemented with foetal calf

serum (15% vol/vol), 2 mM L-glutamine, non-essential amino acids solution (1% vol/vol), penicillin (20 units/ml)/streptomycin (20 mg/ml) and amphotericin B (250 ng/mL), at 37°C in 5% CO_2/21% O_2. All tissue culture reagents were purchased from Sigma Aldrich (Sigma Aldrich, Dorset, UK).

SH-SY5Y cells were transiently transfected with siRNA directed against ECE-1 or ECE-2 using the Amaxa Cell Line Nucleofactor Kit V (Lonza, Basel, Switzerland) using program G-004 on a Nucleofactor device (Lonza, Basel, Switzerland). In brief, cells were cultured for 3-4 days until ~80% confluent and electroporation was performed on ~1.8 x 10^6 cells, which were nucleofected with siRNA targeted against ECE-1 (100 nM) or ECE-2 (30 nM) (Life Technologies, Warrington, UK) and seeded overnight in a 6-well plate. The conditioned medium was removed, centrifuged at 13,000 rpm for 10 minutes to remove any cellular debris, and stored at -80°C until used. The cells were subsequently washed in PBS, detached in PBS without magnesium and chloride (Sigma Aldrich, Dorset, UK), pelleted and lysed in non-denaturing Cell Lytic ™ cell lysis buffer (Sigma Aldrich, Dorset, UK). The samples were centrifuged at 13,000 rpm for 15 min at 4°C and the supernatants aliquoted and stored at -80°C until used. Total protein level was determined using Total Protein Kit (Sigma Aldrich, Dorset, UK) and volumes were adjusted to add an equal amount of total protein per sample for measurement of ECE-1, ECE-2, and α-syn by sandwich ELISA, as described above. Each experiment was repeated in duplicate and performed on at least three separate occasions.

Double immunofluorescent labelling of ECE-1/ECE-2 and α-syn

SH-SY5Y and SH-SY5Y-α-syn cells were grown on poly-L-lysine (PLL)-coated coverslips, washed in PBS x3, fixed in 4% paraformaldehyde for 10 min, further washed in PBS and blocked for 30 min in PBS with 0.5% triton-X100 and 5% horse serum (Millipore, Nottingham, UK). Immunofluorescent labelling was firstly optimised for α-syn using mouse monoclonal α-syn (syn-1) (2.5 µg/ml) (BD, Oxford, UK) as previously described (Miners et al. 2014a). ECE-1 (goat polyclonal anti-ECE-1, R&D systems, Cambridge, UK) and ECE-2 (goat polyclonal ECE-2, R&D systems, Cambridge, UK) immunofluorescence was optimised and a panel of established endolysosomal markers (New England Biolabs, Herts, UK) was used according to the manufacturer's guidelines. For double-immunofluorescence we added antibody pairs for 1 h at RT, followed by Alexa-fluor-488 and -555 (Invitrogen, Life Technologies, Paisley, UK) at 1:1000 for 1 h at RT in the dark. Images were acquired under an x100 objective with a confocal scanning laser microscope.

Proximity ligation assay

An *in-situ* proximity ligation assay was performed using a Duolink kit as per manufacturer's guidelines (Sigma Aldrich, Dorset, UK). In brief, SH-SY5Y cells were grown on PLL-coated coverslips and fixed in paraformaldehyde as above. Mouse monoclonal α-syn (2.5 µg/ml, BD, Oxford, UK) was used with either goat polyclonal ECE-1 (R&D systems, Cambridge, UK) or goat polyclonal ECE-2 (R&D systems, Cambridge, UK). For negative controls one of the antibodies for each pair was omitted. The experiments were repeated in triplicate.

Statistical analysis

Parametric statistical tests were used where possible for group comparisons (this required logarithmic transformation of the data for some cases to obtain a normal distribution). ANOVA with a Bonferroni post-hoc test was used for multiple-group comparisons. For datasets that were not normally distributed even after transformation, a Kruskall-Wallis test with Dunn's test for pairwise intergroup comparisons was used. Pearson analysis was used to assess the correlation between pairs of variables. Statistical tests were performed with the help of SPSS version 23. P-values < 0.05 were considered statistically significant.

Results

ECE-1 and ECE-2 cleave α-syn in vitro

Recombinant human ECE-1 and ECE-2 cleaved full-length recombinant human α-syn resulting in a reduction of monomeric α-syn (~16kda band) in a time- (Figure 1) and dose-dependent (Figure 2) manner. ECE-1 and ECE-2 mediated α-syn cleavage resulted in a greater than 50% reduction in the density of monomeric α-syn at 5 minutes with almost complete cleavage observed at ~ 2hrs (Figure 1). ECE-1 and ECE-2 (50ng – 1.56ng) cleaved human α-syn in a dose-dependent manner (Figure 2). ECE-1 and ECE-2 cleaved α-syn at a mildly acidic pH resulting in a similar time-dependent reduction of monomeric α-syn observed at a neutral pH (Figure 3). In comparison to ECE-1, recombinant human α-syn was not cleaved by recombinant human neprilysin (NEP), human angiotensin-converting enzyme-1 (ACE-1), or human insulin-degrading enzyme (IDE) (Supplementary Figure 1). ECE-mediated cleavage of recombinant human α-syn was inhibited in the presence of phosphoramidon (100µM) (Supplementary Figure 2).

ECE-1 and ECE-2 regulate intracellular and extracellular α-syn level in vitro

To assess the possible functional significance of ECE in the homeostasis of α-syn, we measured the level of α-syn in SH-SY5Y cells following transient siRNA-mediated knock-down of ECE-1 or ECE-2. A modest reduction in the expression of ECE-1 (~25% reduction in ECE-1 protein level as measured by ELISA) resulted in significantly increased endogenous α-syn level, both in the cell lysate (~50% increase in α-syn level as measured by ELISA) and in the surrounding culture medium (~40% increase in α-syn level as measured by ELISA) (Figure 4A-C). siRNA-mediated transient knockdown of ECE-2 caused a ~60% reduction in ECE-2 protein level as measured by ELISA, and produced a significant increase in α-syn in the cell lysate (~30% increase in α-syn level as measured by ELISA). A non-significant increase in α-syn level was also measured in the surrounding medium (~20% increase in α-syn level as measured by ELISA) (Figure 4D-F).

ECE-1 and ECE-2 interact with α-syn within the endolysosomal pathway

Co-localisation of ECE-1 with α-syn was observed in SH-SY5Y cells and SH-SY5Y cells overexpressing human α-syn (SH-SY5Y-α-syn) (Figure 5). α-syn was endogenously expressed throughout the cytoplasm in SH-SY5Y cells (Figure 5A) and was increased in SH-SY5Y-α-syn cells (Figure 5D). ECE-1 was abundantly expressed throughout the cytoplasm (Figure 5B and E) where it co-localised with markers of maturing endosomes (mannose-6-phosphate receptor and Rab 7) (Supplementary Figure 3). ECE-1 also co-localised with α-syn (Figure 5C and F) and the amount of co-localisation between ECE-1 and α-syn appeared visually greater within individual cells in SH-SY5Y-α-syn cells (Figure 5F). ECE-2 was also distributed throughout the cytoplasm but had a more punctate appearance (Figure 6B and E) (a small degree of co-localisation was observed between ECE-2 and markers of early endosomes - early endosome autoantigen 1 (EEA1) and Rab 5 (Supplementary Figure 4)). Merged images show focal points of co-localisation between ECE-2 and α-syn in SHSY-5Y (Figure 6C) and SH-SY5Y-α-syn cells (Figure 6F). To confirm that ECE-1 and ECE-2 were localised within close proximity of α-syn, we used a proximity ligation assay (Figure 7). These images demonstrate co-localisation of both ECEs with α-syn, at a distance of 15nM or less. As with double immunofluorescence studies co-localisation of α-syn with ECE-1 was more apparent than with ECE-2.

ECE-1 and ECE-2 level is reduced in DLB and correlates inversely with α-syn-P129

To determine whether the levels of ECEs are altered in Lewy body disease and potentially contribute to the pathogenesis of DLB we measured ECE-1, ECE-2, α-syn and α-syn-P129 levels in cingulate cortex from DLB

and control brains by ELISA. We showed previously that α-syn-P129 is a reliable indicator of severity of Lewy body pathology (Miners et al. 2014a, Swirski et al. 2014). ECE-1 and ECE-2 levels were significantly reduced in DLB compared to age-matched controls (Figure 8A-B). Reduced ECE-1 and ECE-2 levels correlated inversely with increasing α-syn-P129 level across the cohorts (Figure 8C-D).

Discussion

Endothelin-converting enzymes are expressed throughout the brain and are involved in the hydrolysis of a range of biologically diverse peptides. Alterations in ECE activity have been suggested to be involved in the pathogenesis of Alzheimer's disease, as ECEs were shown to cleave amyloid-β (Aβ) (Eckman et al. 2001), the levels of which are elevated in ECE-knockout mice (Eckman et al. 2003). Here we have shown for the first time that ECEs cleave α-syn and that knockdown of either ECE-1 or ECE-2 increases the amount of both intracellular and extracellular α-syn. We have also shown that ECEs interact with α-syn within the endolysosomal pathway in SH-SY5Y cells. We have shown too that ECE-1 and ECE-2 levels are significantly reduced in cingulate cortex (an area with a predilection for α-syn accumulation in Lewy bodies in DLB (Miners et al. 2014a, Swirski et al. 2014)) in post-mortem brain tissue from people with DLB, and that the concentration of α-syn-P129 (which accounts for ~90% of the α-syn within Lewy bodies (Anderson *et al.* 2006, Fujiwara *et al.* 2002)) correlated with the extent of reduction in ECEs in the same tissue samples. Together these findings suggest a novel role for ECEs in the homeostasis of α-syn and suggest that deficient enzyme activity may contribute to the pathogenesis of DLB, although further work is required.

ECEs are expressed throughout the brain. ECE-1 is expressed abundantly in endothelial cells but has also been identified within non-vascular cells within the CNS such as pyramidal neurons, cerebellar Purkinje cells and astrocytes (Naidoo et al. 2004, Palmer et al. 2010). Of the four isoforms, ECE-1a and ECE-1c are mainly expressed at the plasma membrane whereas ECE-1d and ECE-1b are localised to intracellular compartments including the recycling and late endosomes (Azarani et al. 1998, Muller et al. 2003, Schweizer et al. 1997). ECE-2 is expressed predominantly by pyramidal neurons in the CNS (Palmer *et al.* 2009) and has four isoforms that are expressed intracellularly. ECE-1 and ECE-2 degrades a variety of neuropeptides within endosomes and are responsible for regulating receptor recycling during neuropeptide signalling (Padilla *et al.* 2007, Roosterman *et al.* 2007, Roosterman et al. 2008, Gupta et al. 2015).

It is becoming increasingly clear that disturbances in clearance play a major role in the accumulation of a range of aggregation-prone, disease-related proteins within the brain. Many of the pathways involved in the clearance of α-syn are dysregulated in Lewy body diseases (reviewed in (Deleidi & Maetzler 2012, Ebrahimi-Fakhari et al. 2012, Webb et al. 2003, Xilouri et al. 2013)). There is strong evidence that dysregulation of the ubiquitin-proteosomal pathway and the autophagy-lysosomal system (cathepsin D is an important mediator of degradation of α-syn within the lysosome) (Cullen et al. 2009, Qiao et al. 2008, Sevlever et al. 2008)) are involved in the accumulation of α-syn in Lewy body diseases. Another probable contributor to this accumulation is a reduction in CAPN1 and KLK6, two recently identified α-syn-degrading proteases, the levels of which we recently reported to be reduced within the cingulate cortex in DLB and were inversely correlated with the level of α-syn phosphorylated at Ser129 (Miners et al. 2014a), as was the case for the concentration of ECEs in the present study.

Discrete intracellular pools of α-syn have been detected in early, late and recycling endosomes (Hasegawa *et al.* 2011, Lee *et al.* 2008), multi-vesicular bodies and lysosomes (Boassa *et al.* 2013), autophagic vesicles following chaperone-mediated autophagy (Mak *et al.* 2010), and exosomes (Lee *et al.* 2005). Dysregulation of the endo-lysosomal and autophagic systems, which occurs early in the development of Lewy body diseases, has been implicated in the accumulation, vesicular spread and trans-neuronal propagation of pathogenic α-syn (Luk et al. 2012, Mougenot et al. 2011, Mougenot et al. 2012, Masuda-Suzukake et al. 2013). Our data show that ECEs interact with α-syn within several intracellular locations that impact on the secretion of α-syn, although further work is required to confirm this. Our findings are similar to the observations of Pacheco-Quinto et al., 2013 (Pacheco-Quinto & Eckman 2013) who demonstrated that ECEs degrade discrete pools of vesicular Aβ: ECE-2 cleaved an intracellular pool of Aβ which was produced and degraded within the autophagic and endo-lysosomal pathway whereas ECE-1 (although contributing to both pathways) predominantly degraded a pool of Aβ destined for secretion. A recent study demonstrated that an increase in KLK6, which is synthesised as an inactive zymogen and secreted extracellularly whereupon it is activated and degrades extracellular α-syn (Tatebe *et al.* 2010), could also reduce the transcellular spread of pathogenic α-syn (Yamashiro *et al.* 1997). Further work is required to determine whether ECEs, particularly ECE-1 might influence the transcellular spread and CNS accumulation of α-syn as postulated for KLK6.

In this study, we have shown that ECE-1 and ECE-2 degrade full-length monomeric α-syn at both a neutral and mildly acidic pH in a time- and dose-dependent manner. Over 50% reduction of monomeric α-syn

was observed in the first five minutes with complete degradation observed at 2 hours. This is in-keeping with relatively rapid CTSD (Sevlever et al. 2008) and CAPN-1 mediated degradation of α-syn (Dufty *et al.* 2007) in contrast to slower and incomplete KLK6-mediated cleavage (6-24 hours) (Kasai et al. 2008). To fully understand the potential role of ECE-1 and -2 in the pathogenesis of Lewy body diseases, such as DLB, however, requires further mapping of ECE-mediated α-syn cleavage sites and characterisation of the cleavage fragments within human brain tissue and in animal studies. It has been established that cleavage within the NAC domain reduces the aggregation of full-length wild-type α-syn, as shown for KLK6 (Kasai et al. 2008). C-terminal truncation on the other hand, as shown for CAPN1 (Dufty et al. 2007, Mishizen-Eberz et al. 2005), and MMP-3 (Choi *et al.* 2011, Sung et al. 2005) mediated cleavage of fibrillary α-syn produces fragments that accelerate the aggregation of α-syn. CAPN1-mediated cleavage of α-syn has been postulated to accelerate disease (Higuchi *et al.* 2012, Yamashima 2013) whereas KLK6-mediated cleavage (Spencer *et al.* 2013) and CTSD cleavage (Cullen et al. 2009, Qiao et al. 2008) is protective. It also remains to be determined whether disease-associated mutant forms of α-syn (such as those carrying A53T or A30P mutations), or phosphorylated forms of α-syn, are resistant to ECE-mediated degradation as previously shown for KLK6 and CAPN1 (Iwata et al. 2003, Kasai et al. 2008, Mishizen-Eberz et al. 2003). Lastly, the ability of ECE to cleave such a relatively large substrate (Mr of α-syn is 14kDa) compared to most neuropeptides and beta amyloid (between 1-4 KDa) is also unusual and is not shared by closely related metallo-endopeptidases such as neprilysin and insulin-degrading enzyme.

In conclusion, we present novel evidence that ECEs cleave and degrade α-syn *in vitro* and may contribute to the homeostasis of α-syn and the pathogenesis of Lewy body diseases.

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Conflict of interest The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1. Time course of ECE-1 and ECE-2 cleavage of α-syn *in vitro*. Full-length wild-type recombinant human α-syn (5ng) was incubated with recombinant human ECE-1 and ECE-2 (50ng) in either distilled water or PBS and incubated at 37°C with shaking for 18 hours. Samples were taken at various time-points and transferred to a nitrocellulose membrane followed by western blotting with mouse monoclonal anti-α-syn (syn-1). **A-B** representative western blots of ECE-2 mediated α-syn cleavage performed in distilled water and PBS from a single experiment. **C-D** Bar charts showing the relative band density of monomeric α-syn (~16 kDa), at the different time-points (expressed as a percentage of the control at 0 minutes which was arbitrarily assigned a

value of 100%) up to 2 hours following incubation with ECE-1 and ECE-2. No bands were detected at 4, 6 and 18 hours (not shown). Each experiment was repeated in duplicate and the bars represent the mean ± standard deviation.

Figure 2. Dose-dependent cleavage of α-syn by ECE-1 and ECE-2 *in vitro*. Full-length recombinant α-syn, diluted in distilled water, was incubated with recombinant human ECE-1 and ECE-2 at various concentrations (50 – 1.25ng) in distilled water for 10 minutes before samples were transferred to a nitrocellulose membrane followed by western blotting with mouse monoclonal anti-α-syn (syn-1). **A** representative image showing dose-dependent ECE-1 mediated reduction in monomeric α-syn (~16 kDa) from a single experiment. **B** Bar chart showing the relative band density of monomeric α-syn (~16 kDa) following incubation at the different concentrations of ECE-1 and ECE-2 (expressed as a percentage of the control at 0 minutes which was arbitrarily assigned a value of 100%). Each experiment was repeated in duplicate and the bars represent the mean ± standard deviation.

Figure 3. ECE-1 and ECE-2 cleavage of α-syn at mildy acidic pH *in vitro*. Full-length α-syn was incubated with recombinant human ECE-1 (50ng, pH 6.0) and ECE-2 (50ng, pH 5.5) at a mildly acidic pH. **A-B** representative images showing the time course of monomeric α-syn cleavage by ECE-1 and ECE-2 at pH 6.0 and pH 5.5 respectively from a single experiment. **C** Bar chart showing the relative band density of monomeric α-syn at the different time-points (expressed as a percentage of the control at 0 minutes which was arbitrarily assigned a value of 100%) up to 2 hours. Each experiment was repeated in duplicate and the bars represent the mean ± standard deviation. No bands were detected at 4, 6 and 18 hours (not shown).

Figure 4. ECE-1 and ECE-2 regulate an intracellular and extracellular pool of α-syn in SH-SY5Y cells. A-C siRNA-mediated knockdown of ECE-1 (~25% reduction) resulted in increased α-syn levels in the cell lysate (~50%) and in the cell medium (~40%) of SH-SY5Y cells. **D-F** siRNA-mediated knockdown of ECE-2 (~60% reduction) resulted in increased α-syn levels in the cell lysate (~30%) and, although not significant, increased α-syn level within the cell medium (~20%). ECE-1, ECE-2 and α-syn level were measured by sandwich ELISA in cell lysates adjusted for total protein. Experiments were repeated on at least three separate occasions. $P < 0.05$

Figure 5. ECE-1 co-localised with α-syn in SH-SY5Y neuroblastoma cells overexpressing human wild-type α-syn (SH-SY5Y-α-syn). **A** and **D** show widespread cytoplasmic distribution of α-syn (green) in SH-SY5Y cells which was increased in SH-SY5Y cells stably transfected to overexpress human α-syn (SH-SY5Y-α-syn). **B** and **E** ECE-1 (red) was abundant and had a distinctive perinuclear distribution in both cell lines. **C** and **F** In merged images, ECE-1 co-localised (orange-yellow) with α-syn which appeared visually greater within individual cells in SH-SY5Y-α-syn cells. Images were acquired under a x60 objective with a confocal scanning laser microscope. Nuclei were stained with DAPI (blue). Representative images are from a single experiment. Each experiment was performed independently on at least two separate occasions.

Figure 6. ECE-2 co-localised with α-syn in SH-SY5Y neuroblastoma cells overexpressing human wild-type α-syn (SH-SY5Y-α-syn). A and **D** α-syn (green) showed widespread cytoplasmic distribution in SH-SY5Y cells which was increased in SH-SY5Y cells stably transfected to overexpress human α-syn (SH-SY5Y-α-syn). **B** and **E** ECE-2 (red) had a punctate appearance in both wild-type SH-SY5Y cells and SH-SY5Y-α-syn cells. **C** and **F** Double immunofluorescence revealed foci of co-localisation (orange-yellow) of ECE-2 and α-syn. Images were acquired under a x60 objective with a confocal scanning laser microscope. Nuclei were stained with DAPI (blue). Representative images are from a single experiment. Each experiment was performed independently on at least two separate occasions.

Figure 7. Co-localisation of ECE-1 and ECE-2 with α-syn was confirmed using a proximity ligation assay in SH-SY5Y cells. A Co-localisation of ECE-1 and α-syn was confirmed in SH-SY5Y cells (red dots). **B** Negative control had omission of ECE-1. **C** Co-localisation of ECE-2 and α-syn was confirmed in the same cell line. **D.** Negative control had omission of ECE-2 antibody. Images were acquired under a x60 objective with a confocal scanning laser microscope. Nuclei were stained blue with DAPI. Representative images are from a single experiment. Each experiment was performed independently on at least two separate occasions.

Figure 8. ECE-1 and ECE-2 levels were reduced in human post mortem brain tissue the cingulate cortex in dementia with Lewy bodies (DLB). A-B Reduced ECE-1 and ECE-2 (measured by sandwich ELISA) in the cingulate cortex, a region with a predilection to Lewy body pathology, in DLB. **C-D** Scatterplots showing ECE-

1 and ECE-2 level correlated inversely with the level of α-syn phosphorylated at Ser129 (a marker of Lewy body pathology) measured by sandwich ELISA in the same samples. * $P < 0.05$;** $P < 0.01$; *** $P < 0.001$

Table 1. Study cohort

	DLB (n = 11)	**Control** (n = 15)
Age (y ± SD)	77.7 ± 8.9	80.4 ± 10.0
Gender (F:M)	5:6	3:12
PM delay (h ± SD)	28.2 ± 10.3	30.0 ± 16.8

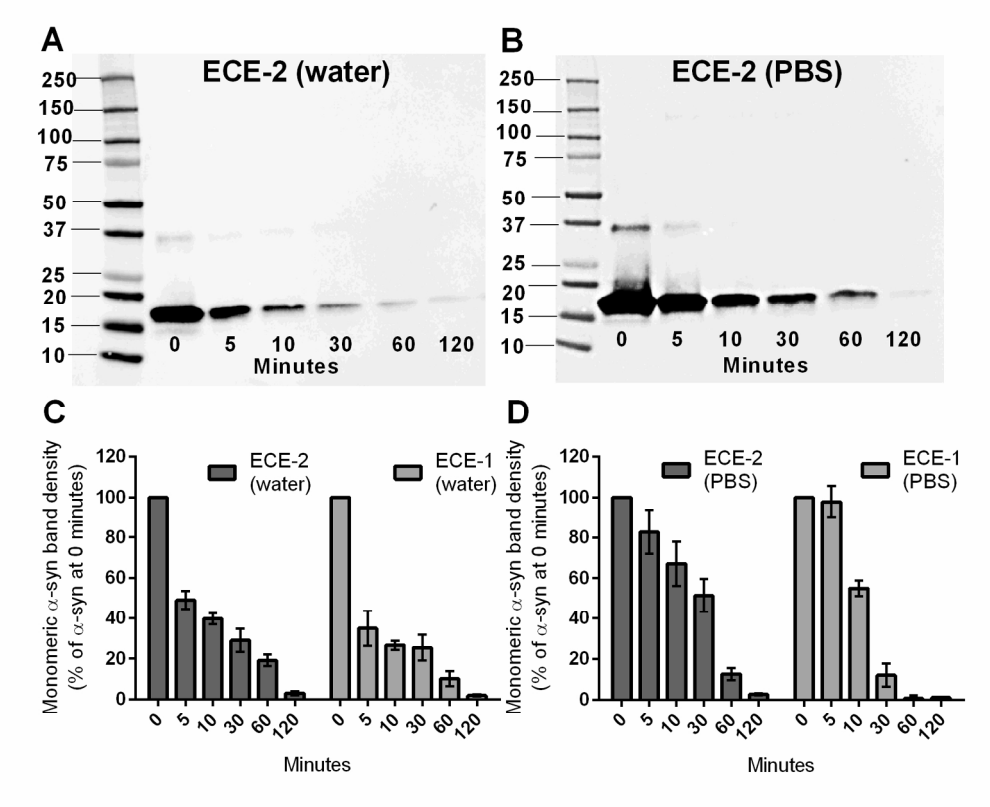

Figure 1

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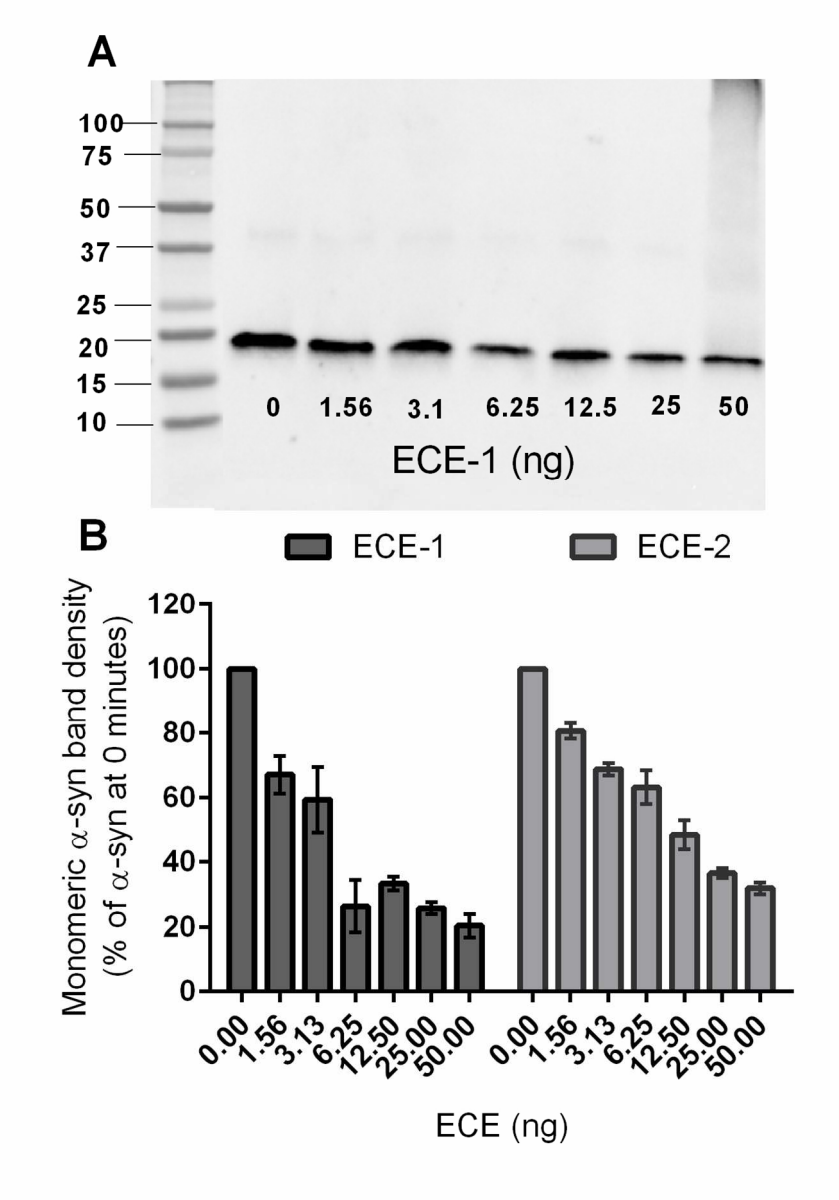

Figure 2

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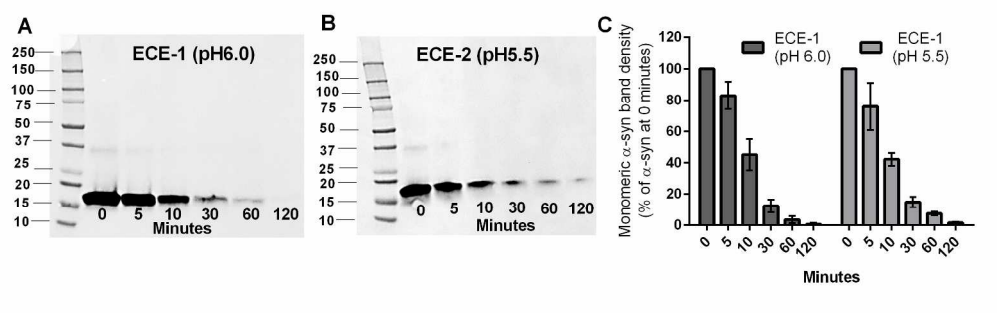

Figure 3

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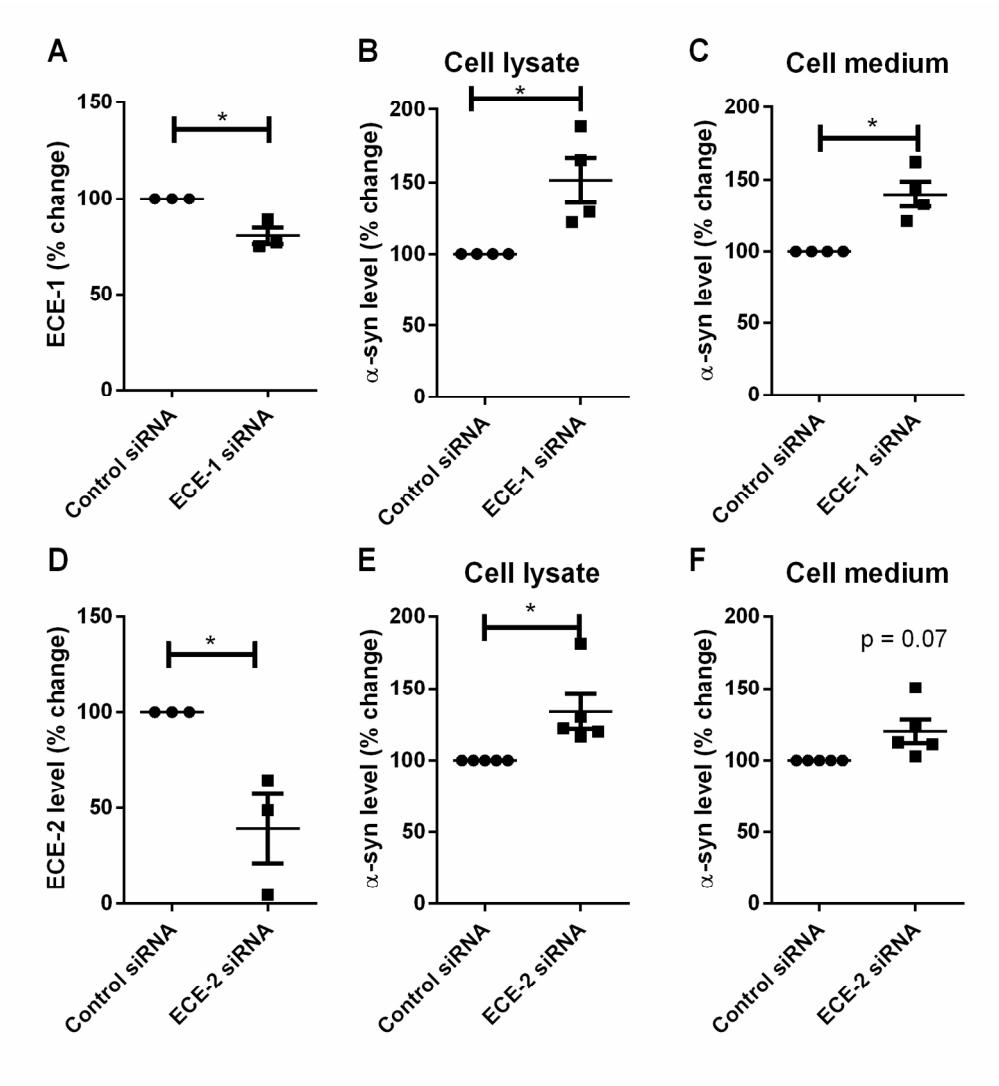


Figure 4

155x167mm (300 x 300 DPI)

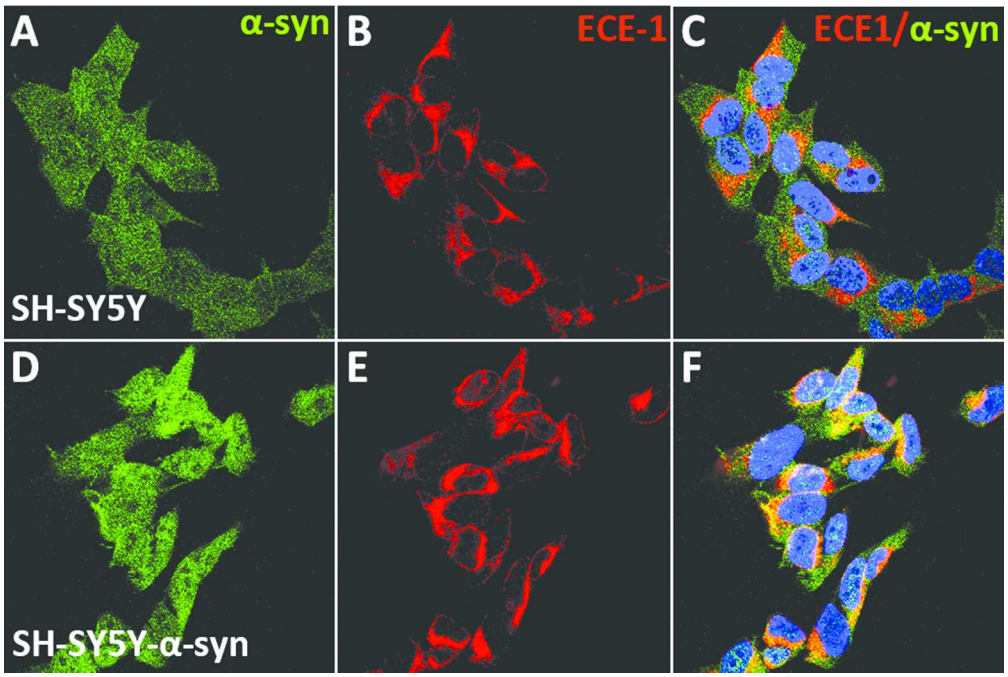


Figure 5

90x60mm (300 x 300 DPI)

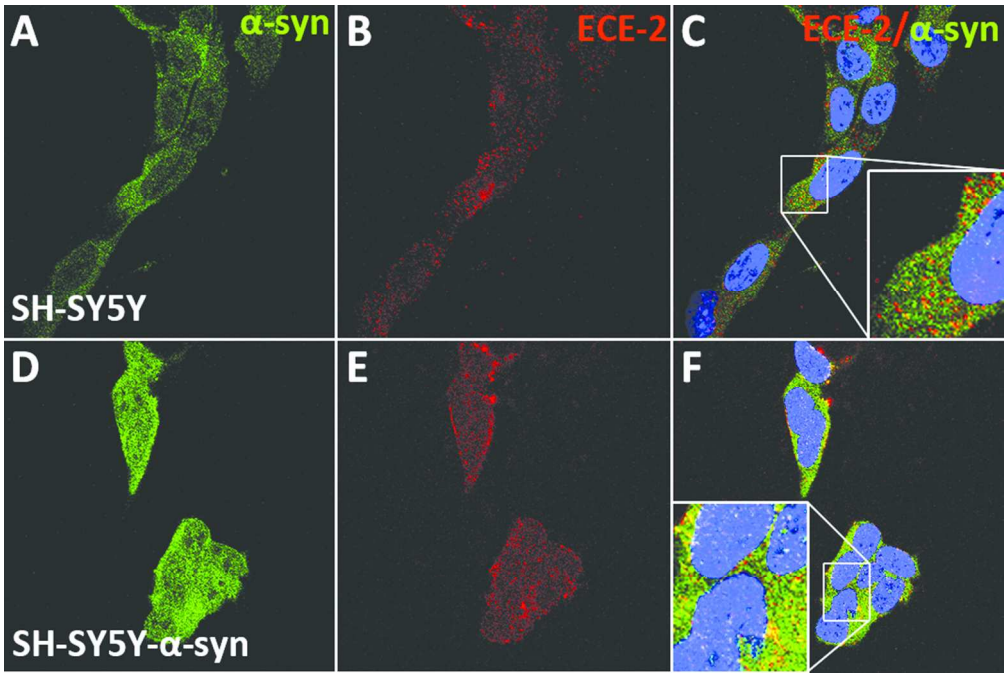


Figure 6

90x60mm (300 x 300 DPI)

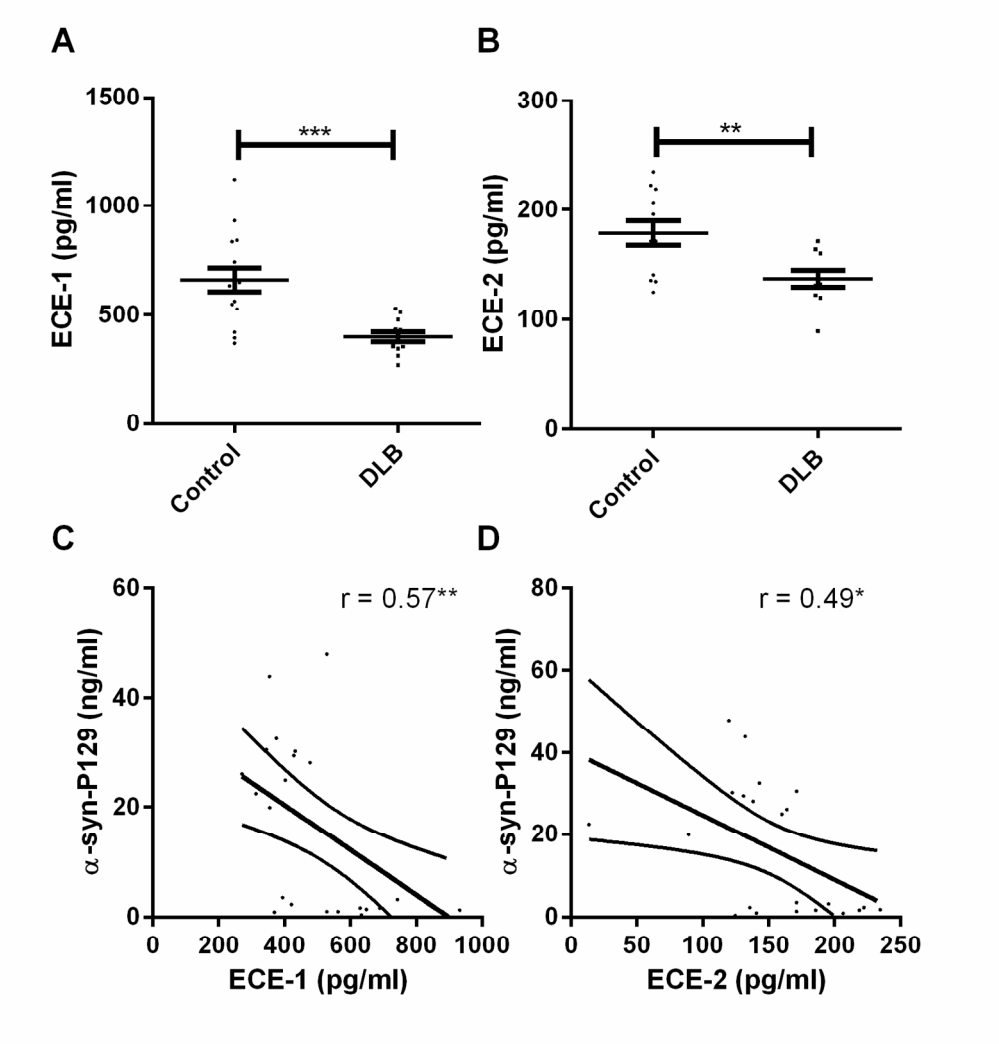
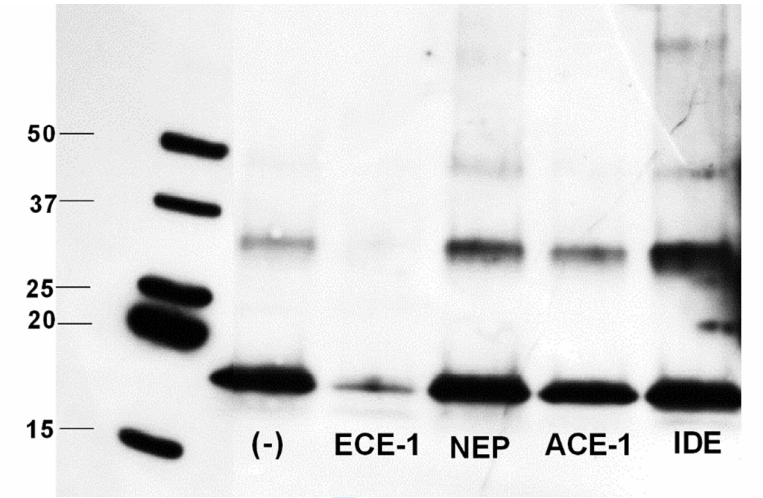


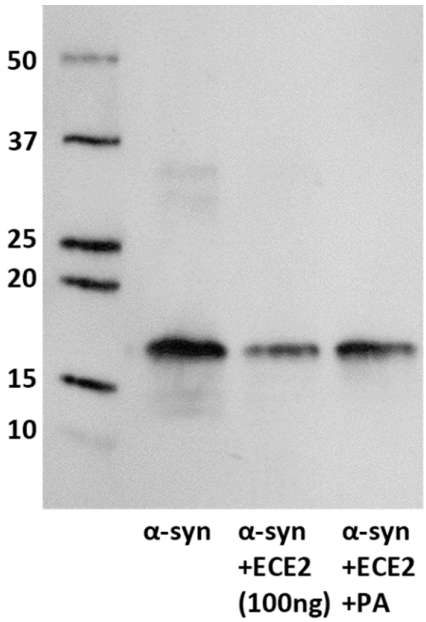
Figure 8

145x152mm (300 x 300 DPI)

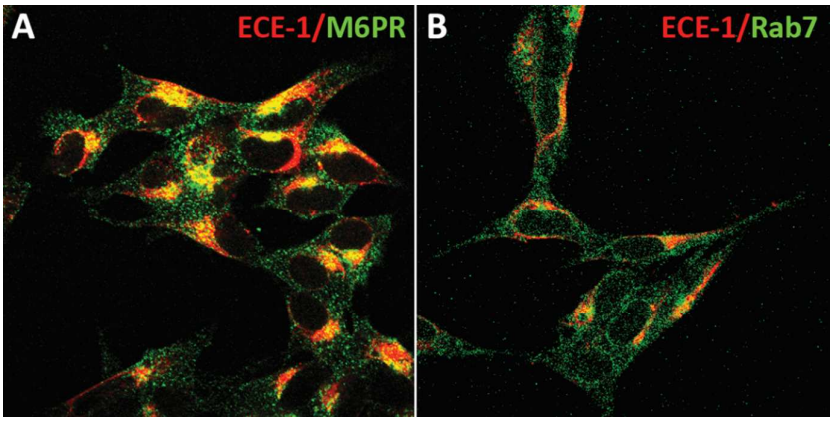
Miners JS et al., Endothelin-converting enzymes degrade α-synuclein and are reduced in dementia with Lewy bodies



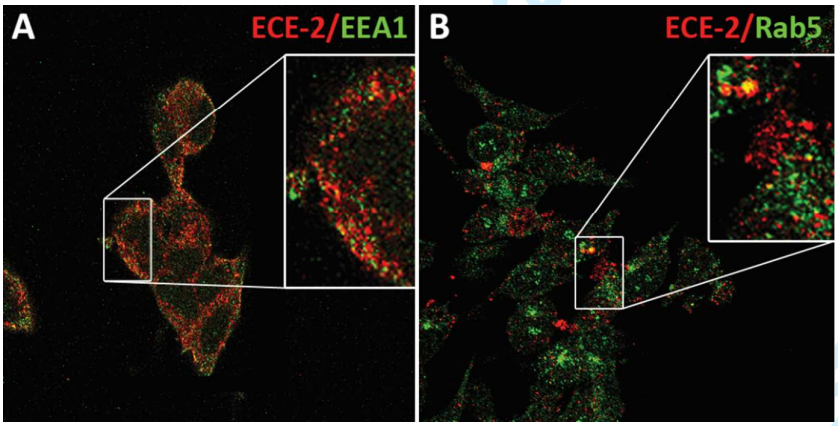
Supplementary Figure 1. Comparison of α-synuclein cleavage *in vitro* by endothelin-converting enzyme-1 (ECE-1), neprilysin (NEP), angiotensin-converting enzyme-1 (ACE-1) and insulin-degrading enzyme (IDE). Full-length wild-type recombinant human α-syn (5ng) was incubated, either alone (-), or with recombinant human ECE-1, NEP, ACE-1, and IDE (all at 50ng) in distilled water at 37°C with shaking for 2 hours. Samples were transferred to a nitrocellulose membrane followed by western blotting with mouse monoclonal anti-α-syn (syn-1). Representative western blot showing full-length monomeric α-syn (~16 kDa). This is a representative image from a single experiment. The experiment was performed independently on two separate occasions.



Supplementary Figure 2. ECE-2 mediated cleavage of α-synuclein (α-syn) is inhibited in the presence of phosphoramidon (PA). Full-length wild-type recombinant human α-syn (5ng) was incubated, either alone, or with recombinant human ECE-2 (100ng), with and without phosphoramidon (PA) (100μM), in distilled water at 37°C with shaking for 2 hours. Samples were transferred to a nitrocellulose membrane followed by western blotting with mouse monoclonal anti-α-syn (syn-1). Representative western blot showing full-length monomeric α-syn (~16 kDa). This is a representative image from a single experiment. The experiment was performed independently on two separate occasions.



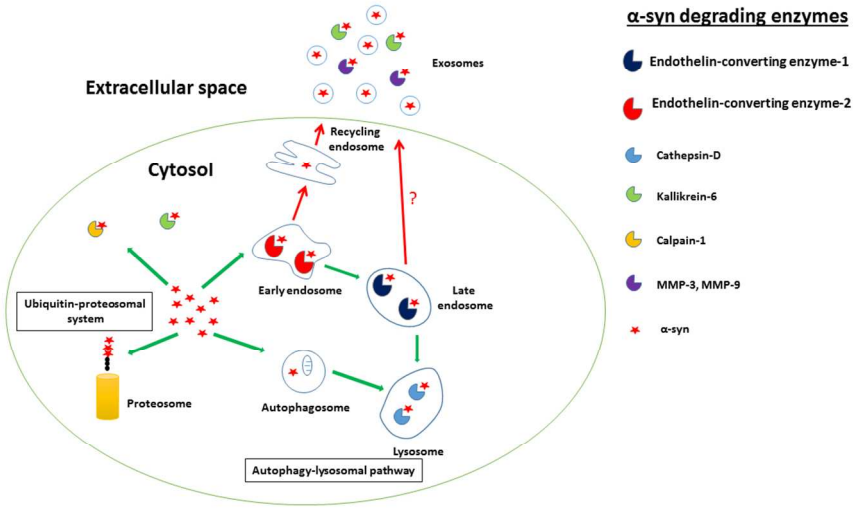
Supplementary Figure 3. ECE-1 co-localised with markers of the 'late/maturing' endo-lysosomal system in SH-SY5Y cells. In merged images ECE-1 (red) co-localised (orange-yellow) with (A) mannose-6 phosphate receptor (M6PR) (green) and (B) Rab7 (green), markers of late and maturing endosomes respectively. Images were acquired under a x100 objective with a confocal scanning laser microscope.



Supplementary Figure 4. ECE-2 co-localised with markers of the 'early' endo-lysosomal system in SH-SY5Y cells. A low level of co-localisation (orange-yellow) was observed between ECE-2 (red) and (A) early endosome antigen 1 (EEA1) (green) and B. Rab5 (green), markers of early endocytosis. Images were acquired under a x100 objective with a confocal scanning laser microscope.

Diagnosis	Age-at-death (y)	Gender	Post-mortem delay	MRC identifier
DLB	68	M	24	BBN_9017
DLB	75	M	53	BBN_9039
DLB	77	M	21	BBN_9064
DLB	97	F	24	BBN_9135
DLB	76	F	33	BBN_9316
DLB	86	M	15.25	BBN_9321
DLB	69	M	38	BBN_9334
DLB	85	F	30	BBN_4198
DLB	79	F	26	BBN_9351
DLB	76	M	26	BBN_9370
DLB	67	F	20	BBN_9384
Control	64	M	12	BBN_8700
Control	75	M	48	BBN_8759
Control	69	M	66	BBN_8779
Control	82	M	3	BBN_8923
Control	72	F	24	BBN_8980
Control	76	M	23	BBN_9028
Control	73	M	35	BBN_9292
Control	90	M	5.5	BBN_9299
Control	93	M	37.75	BBN_9311
Control	94	F	21	BBN_9340
Control	92	M	34.25	BBN_9344
Control	87	M	24	BBN_4205
Control	85	M	30.5	BBN_9354
Control	87	M	47	BBN_4229
Control	68	F	38.75	BBN_9389

Supplementary Table 1. MRC identifiers



338x190mm (96 x 96 DPI)

In this issue. Defective clearance, rather than increased production, most likely contributes to intracellular accumulation of α-synuclein in Lewy body diseases, including dementia with Lewy bodies (DLB) and Parkinson's disease (PD). Several enzymes are capable of degrading α-synuclein (as shown) and are often dysregulated in DLB and PD. Here we show for the first time that recombinant human α-synuclein is cleaved by endothelin-converting enzyme-1 and -2 (ECE-1/-2) and siRNA-mediated knockdown of ECE-1 or -2 increased intracellular and extracellular α-syn within SH-SY5Y cells. ECE-1 and -2 co-localise with discrete pools of intracellular α-synuclein that are implicated in the spread and propagation of Lewy body pathology. Finally, we show that ECE-1 and -2 levels are significantly reduced in post-mortem brain tissue in the cingulate cortex in DLB and correlate inversely with Lewy body pathology.